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RESEARCH ARTICLE

Intestinal Metaplasia and Over-Expression of c-erb2 and p53 in Tissue Adjacent to Dog Gastric Carcinoma

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ABSTRACT

Histological features and genetic profiles of gastric metaplastic tissue are well characterized in humans but not in dogs. The objective of this retrospective study was to better characterize the metaplastic tissue observed adjacent to canine gastric carcinoma. The histological specimens of 91 dogs diagnosed with gastric carcinoma were re-evaluated and Alcian Blue PAS staining at pH 2.5 was performed to find areas of intestinal metaplasia. Metaplasia was histologically classified according to Jass and Filipe classification. From samples with at least one focus of metaplasia, three sections were prepared for histochemical and immunohistochemical staining for p53 and c-erb 2 proteins. 35 of the 91 specimens demonstrated areas of intestinal metaplasia (27% complete and 11% incomplete). Nuclear positive immunolabeling for p53 was detected in 21 out of 35 cases of intestinal metaplasia. Immunohistochemical staining for c-erb 2 was detected in 31 out of 35 cases of intestinal metaplasia. There was a statistically significant correlation between c-erb2 and p53 expression in metaplastic tissue and in the adjacent neoplasia.

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INTRODUCTION

According to the current literature, carcinoma is the most common gastric neoplasm in dogs. Most animals with gastric neoplasia are relatively asymptomatic until the disease is advanced enough to disturb gastric function. Symptoms are frequently mild and a-specific in end-stage patients. Moreover, there is low client compliance in performing several consecutive endoscopies and gastric biopsies to monitor the possible multi-steps progression from gastritis to gastric neoplasia. Therefore, at the time of clinical presentation, tumours are often at an advanced stage resulting in a poor prognosis with limited treatment options (Minami, 2013).

Curative resection is often complicated by diffuse infiltration and a frequently debilitated patient. According to actual literature, there are no validated pre-neoplastic lesions of gastric carcinoma, even if recently a type of hypertrophic gastropathy like Ménétrier disease has been associated with possible predisposition to gastric cancer development (Lecoindre *et al.*, 2012; Munday *et al.*, 2012).

In humans areas of intestinal metaplasia (IM) in the gastric mucosa adjacent to gastric carcinoma are frequently described (Correa *et al.*, 2010). IM is a well-known condition in human pathology as it occurs in response to a chronic mucosal insult and it is considered a precancerous lesion, part of the Correa's cascade, a multi-step progression from chronic gastritis to cancer (Correa, 1992). In human medicine, gastric IM is histologically classified according to Jass and Filipe classification as complete IM (type 1) or incomplete IM (subdivided into type 2 and type 3), according to the various degree of epithelium differentiation toward a nearly complete small intestinal absorbent columnar cells.

In veterinary medicine, the occurrence of IM in response to a chronic mucosal lesion has been reported (Wang *et al.*, 2000; Gualtieri *et al.*, 2006; Rychlik *et al.*, 2009; Gibson *et al.*, 2010) although histological features and clinical behaviour have never been described. A possible correlation between IM and gastric tumour development has never been investigated.

In one of our patient we could observe the progression of IM to dysplasia and mucinous carcinoma

after 2 months. Because of this specific patient and a previous study conducted by the authors about IM and chronic gastritis (Cocci *et al.*, 2008), we started reviewing cases of dog gastric carcinomas (GC) in order to localize areas of IM associated with neoplasia. The first aim of this study was to histologically classify the metaplastic changes present in the mucosa adjacent to canine GC. It has been largely demonstrated that the accumulation of multiple genetic alterations plays a pivotal role in tumour development in many organs (Waraya *et al.*, 2015).

In particular, the inactivation of the onco-suppressor p-53 gene appears to be involved in the carcinogenesis of various neoplasms (Waraya *et al.*, 2015) and is frequently evident in dysplastic epithelium (Carrasco *et al.*, 2011). C-erb2 gene mutated forms promotes neoplastic transformation and its overexpression is involved in the carcinogenesis of mammary and pulmonary ductal carcinoma and of squamous cell carcinoma (Zheng *et al.*, 2010). Overexpression of c-erb2 has also been demonstrated in 2 dog gastric carcinomas (Terragni *et al.*, 2014). The second aim of this study was to investigate the possible neoplastic evolution of metaplastic tissue through the evaluation of p53 and c-erb 2 proteins expression in metaplastic epithelium.

MATERIALS AND METHODS

This retrospective study included 91 dogs presented to the University of Milan between January 2004 and 2012, with histopathological evidence of GC on either endoscopic or surgical biopsies.

The same two pathologists performed all the analysis. The paraffin-embedded samples were classified histologically according to the World Health Organization of Tumours of Domestic Animals guidelines. According to the growth pattern 5 types of GC were found: tubular carcinoma, papillary carcinoma, mucinous carcinoma, signet-ring cell and undifferentiated carcinoma.

Slides were reviewed with the aim to identify metaplastic changes adjacent to neoplastic lesions. Intestinal metaplasia was histologically classified according to Jass and Filipe (Jass *et al.*, 1980). Metaplasia was considered Complete when gastric epithelium resembled the epithelium of the small intestine, with goblet cells secreting acid mucins and absorbent columnar cells with a well-developed brush border, and occasional Paneth cells at the base of the crypts.

IM was classified as Incomplete (type 2) when both goblet cells and columnar cells secreting neutral mucins were observed, in absence of mature absorbent cells. Metaplasia was considered Incomplete (type 3) when cells appear more differentiated towards intestinal epithelium, although with some degree of disorganization in the glandular architecture, with presence of acid mucins secreting cells. From samples with at least one focus of IM, three white sections were prepared for histochemical and immunohistochemical staining.

Alcian Blue PAS staining at pH 2.5 was performed to evaluate the content of acid (intestinal) or neutral (gastric) mucins. The glass slides were deparafinized in xilene and rehydrated in Ethanol, rinsed in distilled water and stained in Alcian Blue Solution for 30 minutes. Slides were rinsed in distilled water and counterstained in nuclear Red Solution for 5 minutes. Washing in running tape water and dehydrating with Ethanol for 5 minutes was then done, followed by clearing with Xilene for 3 minutes, mounting and cover slipping. Strongly acidic sulphated mucosubstances were stained in blue, nuclei in pink to red and cytoplasms in pale pink.

Additional sections were cut on poly-L-lysine coated glass slides and air dried overnight, deparafinized in xylene and dehydrated in descending dilutions of ethanol. For the antigen retrieval, slides were microwaved in 10 mmol/L sodium citrate buffer (pH 6.0) at 10 min intervals for a total of 20 min. Endogenous peroxidase activity was blocked by 10 min of incubation with 3% hydrogen peroxidase at room temperature. The sections were then incubated with monoclonal mouse antibodies p53 (clone Y5 Epitomics) and c-erbB-2 (polyclonal oncoprotein Scy-Tek) for 30 min. Sections were washed with PBS, incubated with biotinylated secondary antibody at room temperature for 30 min. Diaminobenzidine was finally employed as chromogenic and sections were counterstained with Harris Hematoxylin. Sections of canine mammary carcinoma know to express tested antigens were used as positive control. Negative controls were obtained by substituting the primary antibody with an unrelated serum. Areas of gastric carcinoma included in the sections were used as an internal positive control. Sections of normal gastric mucosa were employed as a negative control.

Immunohistochemical evaluation was performed counting positive cells in a total of 100 cells in 5 random fields at 40X magnification. Immunostaining was considered positive for p53 if the reaction took place in the nucleus and positive for c-erb 2 if it took place in the cytoplasm. Immunohistochemical index was calculated as the average of positive cells in 5 random fields at 40X.

Statistical analyses were performed with Graph Pad Prism 6, using Fisher exact test to compare expression indexes with pathological parameters. Correlation among different variables was determined using Spearman rank correlation analysis. $P \leq 0.05$ was considered statistically significant.

RESULTS

Study population characteristics: The median age was 8.73 years (range from 4 to 16 years) with 59 males and 32 females. Specimens came from 22 different breeds.

The most common was the Mixed Breed (41.7%) followed by the Boxer (7.7%), Dalmatian (5.5%), Belgian Shepherd (4.4%) and Newfoundland (4.4%). Dog with IM were 36 with an average age of 10 years. Breeds with IM were Mixed Breed (n=13), Newfoundland (n=3), Belgian Sheppard (n=3), Beagle (n=2), Border Collie (n=2), Dogo Argentino (n=2), Jack Russell Terrier (n=2), Scottish Sheppard (n=1), Siberian Husky (n=1), German Sheppard (n=1), English Setter (n=1), Lagotto (n=1), Labrador (n=1), Dalmatian (n=1), Chow-Chow (n=1), French Boule Dogue (n=1).

Histological examination: Of the 91 cases evaluated, 40 were tubular adenocarcinomas (44%), 5 mucinous carcinomas (5%), 44 signet ring carcinomas (48%) and 2 were undifferentiated carcinomas (2%). The most frequent

locations were the small curvature and the pyloric antrum (33%). A focus of IM was observed in 35 specimens (38%). In agreement with the above-mentioned histological criteria, 25 cases of complete IM (27%) (Fig. 1, Fig. 2) and 10 cases of incomplete IM type 2 (Fig. 3) (11%) were found. IM was associated with tubular and diffuse or signet-ring carcinoma (Table 1).

In the Complete IM, the gastric epithelium appeared "raised" in irregular pseudo villous structures covered by cylindrical gastric epithelial cells frequently together with Alcian Blue PAS positive goblet cells. Absorbent enterocytes with a brush border and non-absorbent columnar epithelium with abundant cytoplasm and basal nucleus were also observed. These cells showed acid mucins secretion with absence of hyperplasia or villiform structures. No type 3 IM was observed. In only one case a previous tissue sample was available for comparison. It showed a complete Intestinal Metaplasia without histological signs of GC.

Immunohistochemistry: Nuclear positive immune-labeling for p53 was detected in 21 out of 35 cases of IM (60%). Immunohistochemical staining for c-erb 2 was detected in the cytoplasm of cells in 31 out of 35 cases of IM (89%). The values of cell immune-expression for the p53 and c-erb 2 antibodies in the areas of IM and in the neoplastic portion are reported in Graph 1 and Graph 2.

Statistics: There was a statistically significant correlation between c-erb2 expression in metaplastic tissue and in the adjacent neoplasia with a P=0.00598 and a R=0.455. There was also a statistically significant correlation between p53 expression in IM tissue and adjacent neoplasia with a P=0.00137 and a R=0.519. There was no correlation between c-erb2 and p53 expression in gastric carcinomas (P=0.52556, R=-0.1119). There was no correlation between c-erb2 and p53 in metaplastic area (P=0.18183, R=-0.2314).

DISCUSSION

To the authors' knowledge the present study proves for the first time in veterinary medicine the spontaneous occurrence and the frequency of complete and incomplete IM in the context of gastric carcinoma in dogs. Previously gastric IM has been only experimentally inducted in stomach of Beagle dogs by combining treatment of oral administration of N-methyl-N'-nito-N-nitrosoguanidine and ranitidine with X-ray irradiation. This was done to create an animal model for scientific research purpose (Wang *et al.*, 2000).

Age and male to female ratio of dogs in our study matched with reported literature data about gastric carcinoma. In literature, a breed predisposition has been reported for the Rough Collie, Staffordshire terrier, Chow-Chow, Bouvier des Flandres and Groenendael and a familial occurrence has been described in Belgian Shepherds. On the contrary, the breed most represented in this study was the mixed breed (41.7%), but this may be explained by different population trends in different countries. The mixed breed was also the one most associated with gastric IM.

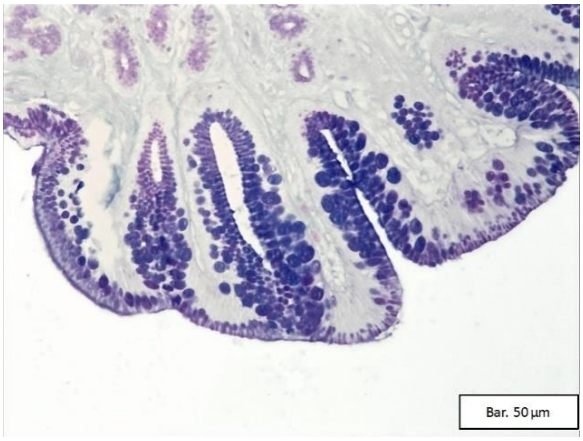


Fig. 1: Complete gastric intestinal metaplasia with prominent goblet cells replacing the normal gastric epithelium. Sialomucines in goblet cells stained in Periodic acid–Schiff–Alcian blue. Bar 50 µm.

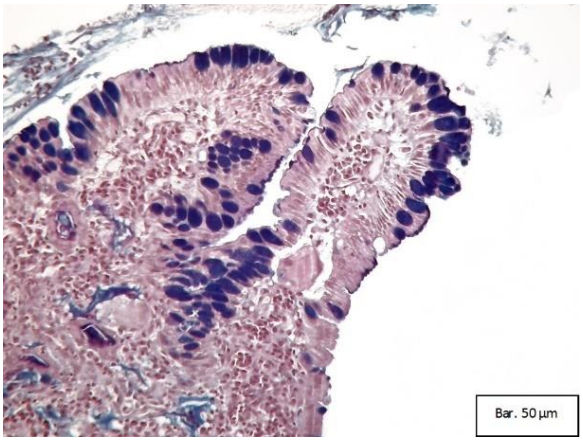


Fig. 2: Complete IM. Gastric antral mucosae lined of columnar cells with brush-border and goblet cells mucinous secernent. Alcian PAS; 10X Bar 50 µm.

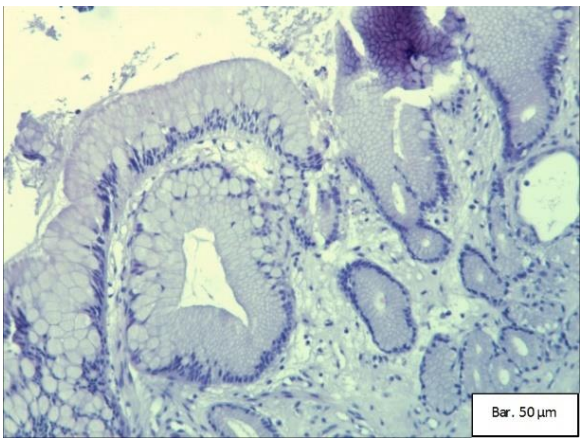


Fig. 3: Incomplete IM: presence of goblet cells between the cylindrical cells lining antral gastric mucosa. No obvious brush border. E.E. 10X. Bar 50 µm.

Table 1: Correlation between tumour pattern and metaplasia pattern

Tumour pattern	IM pattern	n°
Adenocarcinoma	Complete metaplasia	11
Carcinoma signet ring	Incomplete metaplasia	7
Adenocarcinoma	Incomplete metaplasia	2
Carcinoma signet ring	Complete metaplasia	13
Mucinous Adenocarcinoma	Incomplete metaplasia	1
Mucinous Adenocarcinoma	Complete metaplasia	1

IM; Intestinal Metaplasia.

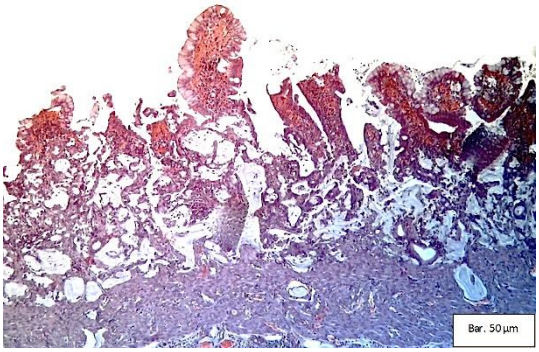


Fig. 4: Complete IM: gastric mucosae with pseudovillous structures, cylindrical epithelial gastric cells and globet acid mucins secreting cells. Mucinous adenocarcinoma infiltranting mucosae and submucosae. Alcian PAS 4X. Bar 50 µm.

Fig. 5: Endoscopic picture of a 9-year old male Rough Collie. Endoscopic diagnosis of gastritis. Histology showed presence of Complete IM on the lesser curvature.

Fig. 6: Endoscopic picture of the same dog two months later. Adenocarcinoma of the lesser curvature of the stomach.

In humans IM derives from gastric stem cells differentiating into cells of the small intestine, in particular into absorbent, goblet and Paneth cells. This sequela generally occurs after a chronic inflammation induced by *Helicobacter pylori* infection (Correa *et al.*, 2010).

Gastric cancer is therefore generally believed to develop through a multistep progression from chronic gastritis to atrophic gastritis, intestinal metaplasia, dysplasia and at last cancer. This kind of gastric carcinogenesis is often called Correa's cascade (Correa 1992). A complete Correa's cascade has not been yet identified in dogs and atrophic gastritis has been rarely reported in this species. Only in Lundehund dogs atrophic gastritis has been associated with possible development of gastric adenocarcinoma (Qvigstad *et al.*, 2008). Unlike human medicine, in which IM is classified into three subtypes, in this study only two patterns were found: Complete type 1-like and the Incomplete type 2-like.

The first is characterized by a pseudo villous aspect of the gastric mucosa with the presence of enterocytes secreting acid mucins, goblet cells and a scarce brush border. The Incomplete type 2 is characterized by irregular lengthening of the gastric foveolae with occasional goblet cells secreting acid mucins, absence of enterocytes and brush border.

The complete IM shared histological features with the human counterpart and, as observed in humans, IM was frequently associated with tubular and diffuse or signet-ring carcinoma. In human medicine, the subtype of IM has a prognostic value. Complete IM is associated with a low risk of gastric carcinoma development, while Type 3 (so-called colic) is strongly associated with carcinoma development. A 10-year follow-up study showed that human patients with type 3 IM were four times more likely to develop gastric carcinoma than patients with type 1 IM (Filipe *et al.*, 1994).

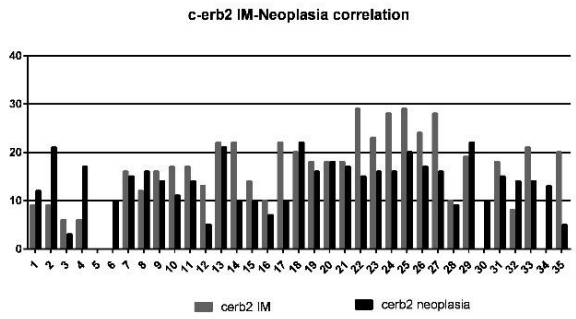
In some IM positive-dogs we observed gastric lesions characterized by non-absorbent columnar epithelium secreting acid mucins in the absence of hyperplasia or villiform structures.

The authors have frequently observed this kind of lesion as an antral reactive phenomenon following biliary reflux (Fig. 4). This agrees with a study from Rychlik, Nieradka *et al.* (2009) in which incomplete IM was found in gastric mucosa biopsies of dogs with inflammatory bowel disease and biliary reflux.

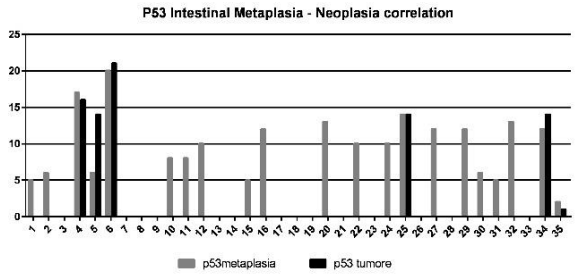
Previous veterinary medicine reports (Gualtieri *et al.*, 2006; Gibson *et al.*, 2010) show that in animals IM is not associated to pathognomonic endoscopic features.

This happens also in human medicine. as conventional endoscopic identification of intestinal metaplasia has a high rate of inter-observer variability and correlates poorly with the histological findings. Intestinal metaplasia usually appears as flat mucosa and shows few morphologic changes. In some rare human patients, IM may be associated with a patchy or villous aspect of gastric mucosa, with whitish plaques or with a homogeneous discoloration (Lin *et al.*, 1999). Nowadays in human and veterinary medicine, endoscopic targeted biopsies of IM can be done after methylene blue gastric staining, a stain able to tinge the normal absorptive epithelium of the small intestine (Calle *et al.*, 2013).

Histological staining of endoscopic or surgical biopsies is currently a reliable exam to identify IM. Unfortunately, in this study it was not possible to evaluate the follow-up biopsies of the subjects examined because most of the specimens came from animals euthanized following the diagnosis of GC. In veterinary medicine the low clients' compliance prevents veterinary surgeons to perform several endoscopies and biopsies to monitor the possible multi-steps progression from gastritis to AG, IM and gastric neoplasia.



Graph 1: Correlation of immunoexpression of c-erb2 protein in intestinal metaplastic lesion and in gastric carcinoma.



Graph 2: Correlation of immunoexpression of p-53 protein in intestinal metaplastic lesion and gastric carcinoma.

Only in one of our patient, a 9-year old male Rough Collie, we were able to detect the multi-step progression of the pathology through serial gastrointestinal endoscopies and biopsies. This dog was at first diagnosed with gastritis associated to complete IM (Fig. 5) and then developed intra-mucosal carcinoma associated to complete IM (Fig. 6). This single case and the results of this work suggest a possible correlation between IM and gastric carcinoma development through a multi-step process resembling the Correa's cascade.

However, it is now impossible for us to affirm that a clear association between IM and GC exists. Further studies are needed to detect a complete Correa's cascade in the canine species and we think that clinicians should consider routine endoscopic monitoring for animal diagnosed with gastric IM. Previous studies carried out on canine gastric carcinoma have pointed out an overexpression of the p53 protein in all types of carcinoma (Hattori, 2004). Carrasco *et al.* (2011) reported an association between degree of tumour infiltration and p53 expression, confirming the usefulness of p53 expression as a prognostic factor.

In our study, the immunohistochemical staining for p53 protein showed a positivity labelling of metaplastic intestinal cells adjacent to gastric carcinoma in 60% of our cases (Adj R2=0.33). A positive correlation was found between p53 expression in metaplastic tissue and in the adjacent neoplasia (P=0.00137, R=0.519). Positive immunolabeling was detected only in biopsies with Complete IM, where only goblet cells were present. Moreover, it was noteworthy that IM cells positive for p53 were often contiguous to carcinomatous areas leading to the hypothesis of malignant progression from IM to gastric carcinoma.

A positive immunolabeling was also found for c-erb2 protein in the 97.2% of metaplastic lesions (Adj R2=0.19). A positive correlation between c-erb2 expression in metaplastic tissue and in the adjacent neoplasia was found (P=0.00598, R=0.455). Kanaya *et al.* (2002) associate the overexpression of c-erb 2 with the degree of malignancy and tumour infiltration in numerous canine epithelial neoplasia

Moreover, in human medicine, the expression of c-erb 2 has been recognized as responsible for the initial stage of mammary tumours. The overexpression of c-erb2 proteins in the cells of the basal layer of ameloblastomas promotes the malignant transformation of keratinocytes (Groves *et al.*, 1992). The identification of c-erb2 in metaplastic stomach cells of our specimens suggested its involvement in precancerous lesions as already reported by Rungsipapat *et al.* (2000).

However, we detected no correlation between c-erb2 and p53 expression in gastric carcinomas (P =0.52556, R=-0.1119). We detected no correlation between c-erb2 and p53 in metaplastic areas (P=0.181836, R=-0.2314). Further studies are needed, possibly with a control group, to better evaluate the relationship between these two proteins expression.

Conclusions: From the study it was concluded that Complete and Incomplete IM and overexpression of p53 and c-erb 2 have a close relationship with gastric carcinoma. Further studies on a larger number of cases and clinical follow-up are necessary to define with greater scientific accuracy the role of intestinal metaplasia in canine gastric carcinoma development.

Authors contribution: All authors have contributed to the preparation and analysis of this article and approved the final version of it.

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